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PRINCIPAL INVESTIGATOR: Michelle A. Carmell
Gregory J. Hannon, Ph.D.

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

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Whole Genome Epigenetics

Michelle A. Carmell  
Gregory J. Hannon, Ph.D.

Cold Spring Harbor Laboratory  
Cold Spring Harbor, New York 11724

E-Mail: carmell@cshl.edu

U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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An understanding of the cancer cell begins with knowledge of the genetic alterations that lead to neoplastic transformation. Much progress has been made in identifying areas of deletion, amplification and mutation in tumors. However, this is only part of the picture. Increasingly, we are learning that epigenetic changes, that is, changes in chromatin structure, are critically important in regulation cellular gene expression.

Recently, several labs have published manuscripts identifying RNA interference as being crucial for the establishment of such epigenetic changes in species as diverse as Drosophila, plants, and the fission yeast S. pombe. This knowledge presented a fantastic opportunity not only to study epigenetic changes, but to actually selectively create epigenetic changes by creating germline transgenic mice in which a target gene has been silenced by RNAi.

After the demonstration that RNAi in mammalian cells can be mediated by vectors encoding short, hairpin RNAs (shRNAs), we sought to develop a system by which to create transgenic mice using this technology. We demonstrated that a stable, heritable RNAi trigger in the form of a short hairpin was successfully passed through the mouse germline. As well as demonstrating a technique that can be applied to any gene of interest, we have created gene knock-down mouse models for the Mcl-1 DNA glycosylase involved in DNA repair pathways, and for p53. These observations open the way to the use of RNAi as a complement to standard knock-out methodologies and provides a means to rapidly assess the consequences of suppressing a gene of interest in a living animal. The vision driving the creation of this technology was one of eventual RNAi-based therapeutics. One could particularly imagine silencing oncogenes in cancer cells to attenuate their tumorigenic tendencies.
Introduction:
An understanding of the cancer cell begins with knowledge of the genetic alterations that lead to neoplastic transformation. Much progress has been made in identifying areas of deletion, amplification and mutation in tumors. However, this is only part of the picture. Increasingly, we are learning that epigenetic changes are critically important in regulating cellular gene expression.

Epigenetic is defined as a change in gene expression that is heritable in cis with a particular allele but that is not specified by an alteration in the DNA sequence of the gene. One mechanistic basis of epigenetic inheritance in mammals is chemical modification of the DNA, specifically the addition of methyl groups that correlate with changes in chromatin structure, and consequently gene expression.

My interest at the time of the writing of this proposal was to examine such epigenetic changes occurring in cancer cells. The eventual goal was the creation of a screening method for identifying, on a patient-to-patient basis, the epigenetic lesions in a particular tumor. This type of technology would allow the clinician to tailor treatment regimens on a per patient basis.

During the time that I was thinking about these types of questions, several labs published manuscripts identifying RNA interference (RNAi) as being crucial for the establishment of such epigenetic changes in species as diverse as Drosophila, plants, and the fission yeast S. pombe. Knowing the high degree of conservation of RNAi-like silencing phenomena, and the recent demonstration that RNAi functions in mammalian systems, I was immediately interested in the role RNAi plays in establishment of the epigenome of mammals. When I realized that not only could I study epigenetic changes, but I could actually selectively create epigenetic changes, I became pulled down a slightly different avenue. I began a project aimed at creating germline transgenic mice in which a target gene has been silenced by RNAi. The vision driving the creation of this technology was one of RNAi-based therapeutics. One could particularly imagine silencing oncogenes in cancer cells to attenuate their tumorigenic tendencies.
Body:
After the demonstration that RNAi in mammalian cells can be mediated by vectors encoding short, hairpin RNAs (shRNAs)\(^1\), we sought to develop a system by which to create transgenic mice using this technology. We demonstrate that a stable, heritable RNAi trigger in the form of a short hairpin was successfully passed through the mouse germline (please see Appendix; Carmell et al.). We observed specific suppression of a target gene at the level of mRNA and protein in multiple tissues. These observations open the way to the use of RNAi as a complement to standard knock-out methodologies and provides a means to rapidly assess the consequences of suppressing a gene of interest in a living animal.

Our approach entailed verifying the presence of the shRNA and its activity toward a target gene in cultured embryonic stem (ES) cells and then asking whether those cells retained suppression in a chimeric animal in vivo. We also hoped to test whether such cells could pass a functional RNAi trigger through the mouse germline. For these studies, we chose to examine a novel gene, Neil1, which is proposed to have a role in DNA repair. The *neil* genes are a newly discovered family of mammalian DNA-N-glycosylases related to the Fpg/Nei family from *E. coli*. Neil1 recognizes and removes a wide spectrum of oxidized pyrimidines and ring-opened purines from DNA\(^2,3\).

Oxidative damage accounts for 10,000 DNA lesions per cell per day in humans and is thought to contribute to carcinogenesis, aging, and tissue damage following ischemia\(^4,5\). Oxidative DNA damage includes abasic sites, strand breaks, and at least twenty oxidized bases, many of which are cytotoxic or pro-mutagenic\(^6\). DNA N-glycosylases initiate the Base Excision Repair (BER) pathway by recognizing specific bases in DNA, and cleaving the sugar base bond to release the damaged base. It has been reported that normal breast tissues from cancer patients have a significantly higher level of oxidative DNA damage than those of women who do not have breast cancer\(^7\). Thus, not only does the Neil-1 knockdown mouse serve as a demonstration of a new technology, it opens a new avenue for the study of DNA repair enzymes.

In addition to the Neil-1 knockdown mice, we have created p53-deficient mice through the use of the same hairpin-based technology. The p53 hairpins that I have used have been validated by Hemann et al.\(^8\), and shown to produce distinct tumor types in vivo when used in the context of reconstituted bone marrow. These p53-knockdown transgenic mice are currently being characterized.
Key Research Accomplishments:

- Establishment of a generally applicable technique for creating germline transgenic mice in which a target gene has been silenced by RNAi
- Establishment of Neil-1 DNA-N-glycosylase knock-down mouse line
- Establishment of several p53 knock-down mouse lines
Reportable Outcomes:

Manuscripts:
Carmell MA, Xuan Z, Zhang MQ, Hannon GJ.
The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev. 2002 Nov 1;16(21):2733-42. Review.

Carmell MA, Zhang L, Conklin DS, Hannon GJ, Rosenuquist TA.

Emily Bernstein, Sang Yong Kim, Michelle Carmell, Elizabeth Murchison, Heather Alcorn, Mamie Z. Lee, Aleea A. Mills, Stephen J. Elledge, Katherine V. Anderson and Gregory J. Hannon. Dicer is essential for mammalian development. Submitted.

Presentations:
Xenogen Biosciences, March 2003

Animal Models:
Neil-1 DNA glycosylase gene knock-down mice for use in study of DNA repair
P53 gene knock-down mice for use in studies of tumorigenesis
Conclusions:
Although I have deviated from my original statement of work, I feel that this has been an extremely productive year, and the training I have received has been of great importance. I am addressing my interest in epigenetics, and have also been able to maintain the ultimate goal of the understanding and treatment of human cancers. I believe that the fields of RNAi and tumor therapy will converge rapidly, and that the technology that I helped to develop will be of crucial importance in the development of mouse models of human cancer.
References:


Appendices:

Carmell et al. Germline transmission of RNAi in mice. (2 pgs total)
Germline of RNAi in mice

Michelle A. Carmell1,2, Ligun Zhang3, Douglas S. Conklin1, Gregory J. Hannon1 and Thomas A. Rosenquist3,4

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MicroRNA molecules (miRNAs) are small, noncoding RNA molecules that have been found in a diverse array of eukaryotes, including mammals. miRNA precursors share a characteristic secondary structure, forming short 'hairpin' RNAs. Genetic and biochemical studies have indicated that miRNAs are processed to their mature forms by Dicer, an RNase III family nuclease, and function through RNA-mediated interference (RNAi) and related pathways to regulate the expression of target genes (reviewed in refs. 1,2). Recently, we and others (reviewed in ref. 3) have remodeled miRNAs to permit experimental manipulation of gene expression in mammalian cells and have dubbed these synthetic silencing triggers 'short hairpin RNAs' (shRNAs). Silencing by shRNAs requires the RNAi machinery and correlates with the production of small interfering RNAs (siRNAs), which are a signature of RNAi.

Expression of shRNAs can elicit either transient or stable silencing, depending upon whether the expression cassette is integrated into the genome of the recipient cultured cell (reviewed in ref. 3). shRNA expression vectors also induce gene silencing in adult mice following transient delivery4. However, for shRNAs to be a viable genetic tool in mice, stable manipulation of gene expression is essential. Hemann and colleagues5 have demonstrated long-term suppression of gene expression in vivo following retroviral delivery of shRNA-expression cassettes to hematopoietic stem cells. Here we sought to test whether shRNA-expression cassettes that were passed through the mouse germline could enforce heritable gene silencing.

We began by taking standard transgenesis approaches6 using shRNAs directed against a variety of targets with expected phenotypes, including the genes encoding tyrosinase (albino), myosin VIIa (shaker), Bmp-5 (crinkled ears), Hox a-10 (limb defects), homogentisate 1,2-dioxogenase (urine turns black upon exposure to air), Hairless (hair loss) and melanocortin 1 receptor (yellow). Three constructs per gene were linearized and injected into pronuclei to produce transgenic founder animals. Although we noted the presence of the transgene in some animals, virtually none showed a distinct or reproducible phenotype that was expected for a hypomorphic allele of the targeted gene.

Therefore, we decided to take another approach: verifying the presence of the shRNA and its activity toward a target gene in cultured embryonic stem (ES) cells and then asking whether those cells retained suppression in a chimera in vivo. We also planned to test whether such cells could pass a functional RNAi-inducing construct through the mouse germline. For these studies, we chose to examine a novel gene, Neil1, which is proposed to have a role in DNA repair. Oxidative damage accounts for 10,000 DNA lesions per cell per day in humans and is thought to contribute to carcinogenesis, aging and tissue damage following ischemia8,9. Oxidative DNA damage includes abasic sites, strand breaks and at least 20 oxidized bases, many of which are cytotoxic or pro-mutagenic10. DNA N-glycosylases initiate the base excision repair pathway by recognizing specific bases in DNA and cleaving the sugar base bond to release the damaged base11.

The Neil genes are a newly discovered family of mammalian DNA N-glycosylases related to the Fpg/Nei family of proteins from Escherichia coli12,13 (T.A.R., E. Zaika, A.S. Fernandez, D.O. Zharkov, H. Miller and A.P. Grollman, submitted). Neil1 recognizes and removes a wide spectrum of oxidized pyrimidines and ring-opened purines from DNA, including thymine glycol (Tg), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formidopyrimidine (FapyA)14 (T.A.R., E. Zaika, A.S. Fernandez, D.O. Zharkov, H. Miller and A.P. Grollman, submitted). Tg, FapyG and FapyA are among the most prevalent oxidized bases produced by ionizing radiation15 and can block replicative DNA polymerases, which can, in turn, cause cell death16,17.

Fig. 1 Heritable repression of Neil1 expression by RNAi in several tissues. a, Expression of Neil1 mRNA in the livers of three mice containing the Neil1 shRNA transgene (shRNA-positive) or three siblings lacking the transgene (shRNA-negative) was assayed by RT-PCR (top row is Neil1). An RT-PCR of β-actin was done to ensure that equal quantities of mRNAs were tested for each mouse (second row). Expression of the neomycin resistance gene (neo), carried on the shRNA vector, was tested similarly (third row). Finally, the mice were genotyped using genomic DNA that was PCR-amplified with vector-specific primers (bottom row). Similar studies were performed in the b, heart and c, spleen. Animal procedures have been approved by the SUNY, Stony Brook Institutional Animal Care and Use Committee (IACUC).
**brief communications**

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Fig. 2 Reduction in Neil1 protein correlates with the presence of siRNAs. **a,** Expression of Neil1 protein was examined in protein extracts from the livers of mice carrying the shRNA transgene (shRNA-positive) or siblings lacking the transgene (shRNA-negative) by western blotting with Neil1-specific antiserum. A western blot for PCNA was used to standardize loading. **b,** The presence of siRNAs in RNA derived from the livers of transgenic mice as assayed by northern blotting using a 300 nt probe, part of which was complementary to the shRNA sequence. We note siRNAs only in mice transgenic for the shRNA expression cassette.

The Nh1 and Oggl glycosylases each remove subsets of oxidized DNA bases that overlap with substrates of Neil1 (refs. 16–18). However, mice with null mutations in either Nh1 (refs. 19,20) or Oggl (refs. 21,22) are viable, raising the possibility that Neil1 activity tempers the loss of Nh1 or Oggl. Recently, a residual Tg-DNA glycosylase activity in Nh1−/− mice has been identified as Neil1 (ref. 23).

We constructed a single shRNA expression vector targeting a sequence near the 5’ end of the Neil1 coding region. This vector was introduced into mouse embryonic stem cells by electroporation, and individual stable integrants were tested for expression of the Neil1 protein (detailed procedures are available at http://www.cshl.edu/public/SCIENCE/hannon.html). The majority of cell lines showed an ~80% reduction in Neil1 protein, which correlated with a similar change in levels of Neil1 mRNA (data not shown). These cells showed an approximately two-fold increase in their sensitivity to ionizing radiation (T.A.R., E. Zaike, A.S. Fernandes, D. O. Zharkov, H. Miller and A. P. Grollman, submitted), consistent with a role for Neil1 in DNA repair. Two independent ES cell lines were injected into BL/6 blastocysts, and several high percentage chimeras were obtained. These chimeras were out-crossed, and germline transmission of the shRNA-expression construct was noted in numerous F1 progeny (13/27 for one line and 12/26 for the other).

To determine whether the silencing of Neil1 that had been observed in ES cells was transmitted faithfully, we examined Neil1 mRNA and protein levels. Both were reduced by approximately the same extent that had been observed in the engineered ES cells (Figs. 1,2). Consistent with this having occurred through the RNAi pathway, we detected the presence of siRNAs corresponding to the shRNA sequence in F1 animals that carry the shRNA expression vector but not in those that lack the vector (Fig. 2b).

The aforementioned data demonstrate that shRNAs can be used to create germline transgenic mice in which RNAi has silenced a target gene. These observations open the door to using of RNAi as a complement to standard knock-out methodologies and provide a means to rapidly assess the consequences of suppressing a gene of interest in a living animal. Coupled with activator-dependent U6 promoters (P. Padisson, J. Du, E. Julien, W. Herr and G.J.H., unpublished data), the use of shRNAs will ultimately provide methods for tissue-specific, inducible and reversible suppression of gene expression in mice.

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1. Cold Spring Harbor Laboratory, Watson School of Biological Sciences, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA. 2. Program in Genetics, State University of New York at Stony Brook, Stony Brook, New York 11794, USA. 3. University Transgenic Mouse Facility, State University of New York, Stony Brook, New York 11794, USA. 4. Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794, USA.

**Competing interests statement**

The authors declare that they have no competing financial interests.

Correspondence should be addressed to T.A.R.

*E-mail: rosenquitt@pharm.sunysb.edu*

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